IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application No. :

U.S. National Serial No. :

Filed :

PCT International Application No. : PCT/FR99/02913

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Date: June 4, 2001

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Multivalent T.d.Polio vaccine against at least diphtheria, poliomyelitis and tetanus

The present invention relates to a novel multivalent vaccine against at least diphtheria, poliomyelitis and tetanus, intended to be used in primary immunization and as an immunization booster in an individual who has already undergone primary immunization or is sensitized.

State of the art

Diphtheria is due to *Corynebacterium diphtheriae*. The transmission is essentially direct via the respiratory pathways. The clinical symptoms of the respiratory form are a product of two mechanisms. The proliferation of the bacteria at the portal of entry determines the local symptomatology, conventionally pharyngeal (pseudomembranous angina), sometimes laryngeal (croup). The diffusion of the exotoxin of certain strains is responsible for malignant angina and/or for visceral complications (polyneuritis, myocarditis, malignant Marfan syndrome). Many serological studies, carried out recently in most developed countries, have shown that, depending on age, sex and local epidemic or immunization history, 20 to 80% of inhabitants are no longer, today, correctly immunized; and that individuals over the age of 50 and females are particularly vulnerable (Rappuoli *et al.*, Vaccine 11, 576-577, 1993. Simonsen *et al.*, Acta Pathol. Microbiol. Immunol. Scand, 95, 225-231, 1987. Wirz *et al.*, Vaccine, 13, 771-773, 1995). This vulnerability has been revealed over the last twenty years by the sometimes dramatic resurgence of diphtheria in developed countries (Galazka *et al.*, Eur. J. Epidemiol, 11, 107-117, 1995).

Tetanus is due to *Clostridium tetani*. The spore form, which is essentially telluric, introduced by breaching the organism (wound, bite/sting, burn or minor lesion), gives rise to the vegetative form. This

form secretes an endotoxin, tetanospasmin. Bacterial lysis causes diffusion thereof. The toxin, which is neurotropic, determines the symptomatology, i.e. an initial trismus and muscle contractions. Without treatment, the evolution is fatal through asphyxia or syncope. Immunization with toxoid remains the only riposte. The immunity acquired with primary immunization decreases over time and requires boosters. The absence or loss of the immunity with age has been noted in the American population for decades, by several serological investigations (Hilton *et al.*, Ann. Intern. Med., 115, 32-33, 1991). In Europe, serological investigations have given equivalent results (Kjeldsen *et al.*, Scand. J. Infect. Dis., 20, 177-185, 1988). It is therefore clear that many adults, particularly those over 50 years old and women, have become, or have always been, vulnerable to tetanus, and require immunization with toxoid.

Poliomyelitis is due to the poliovirus, belonging to the enterovirus group. Three serotypes exist, named type 1, type 2 and type 3. The transmission is essentially fecal-oral, direct or indirect, and sometimes oral-oral. The virus has an intestinal, muscular, meningeal and nervous tropism. The infection is, in general, inconspicuous or mild. This "poliomyelitis infection" is immunizing, but without cross-immunity between types, and is contagious for several weeks. It gives rise exceptionally (1‰ to 1% of infections depending on age and type) to meningeal or paralytic (acute flaccid paralysis) forms. This "poliomyelitis disease" is always serious since there is no etiological curative treatment. When it is not lethal through asphyxia (2 to 10% depending on age and type), it is followed by disabling ventilatory or muscular after-effects. Human exchanges, which are increasingly numerous, between countries endemic for poliomyelitis and countries free of poliomyelitis expose the latter to the risks of importing and of reintroducing wild-type strains. In the last few years,

despite a high level of immunization coverage and a very low worldwide incidence of the disease, imported cases and epidemics have appeared in Europe, in North America and in the Middle East (WHO, Relevé Epidémiologique Hebdomadaire [Weekly Epidemiological Record], 29, 220-221, 1996). While waiting for the disease to be eliminated, the many human exchanges between the countries endemic for the disease and the countries free of the disease therefore risk causing, in any area of the world, outbreaks due to imported wild-type strains. In order to reduce to a minimum the propagation of the disease during exacerbations of this type, it is important that all countries, including those in which there are no longer any reports of a single case of acute poliomyelitis, maintain a high level of immunization coverage throughout the population.

The diphtheria and tetanus vaccines, which were developed by Ramon in the 1930s, are based on toxoids. They can be obtained through the detoxifying action of formaldehyde on a concentrate of *Corynebacterium diphtheriae* or *Clostridium tetani* culture, and then purification. The respective immunizing activities thereof are assessed *in vitro* and *in vivo*. A floculation assay measures the amount of toxoid, expressed in floculation units (Lf) per dose (Lyng *et al.*, J. Biol. Stand., 15 27-37, 1987: J. Lyng. Biologicals, 18, 11-17, 1990).

The tetanus toxoid is sometimes administered alone. However, historically this toxoid has always been combined, at least with the diphtheria toxoid. In general, primary tetanus and diphtheria immunization is carried out during the first year of life in 3 doses (WHO, WHO/EP/GEN, 95.3, 1995). According to country, a booster dose is administered during the second year and/or between 4 and 10 years of age. Sometimes, a booster is also carried out between 11 and 18 years of age. In addition, since 1987 the WHO has recommended immunizing women of child-bearing age in developing

countries with tetanus toxoid, using 3 primary immunization doses and then 1 booster dose 1 and 5 years afterwards.

The amount of tetanus toxoid (T) per immunization dose varies, according to country, from 5 to 20 Lf for one dose of 0.5 ml as a primary immunization or as a booster. The European Pharmacopeia recommends an activity of at least 20 IU.

The most widely and the oldest administered amount of diphtheria toxoid (D) per immunization dose is that which exists in the pediatric primary immunization combinations: it ranges from 12 to 50 Lf for one dose of 0.5 ml (Galazka *et al.*, Vaccine, 14, 845-857, 1996).

For an immunization booster, the decrease in the amount of diphtheria toxoid (d) has now also become generalized. The amount is generally about 1/10 of the pediatric amount. It is thus set at 2 Lf for one dose of 0.5 ml in the US and in Canada, and is recommended as a booster from the age of 7 (Edsall *et al.*, Am. J. Hyg, 53, 283-295, 1951). In Europe, the amount of toxoid and the youngest administration age change according to country. The amount is not set because the only condition imposed by the European Pharmacopeia relates not to the amount but to the activity: it must be at least 2 IU per dose of 0.5 ml.

For booster vaccines intended for an adult population, the diphtheria toxoid is generally also combined with the tetanus toxoid. It is known that this combination slightly decreases the tolerance occurring for the tetanus toxoid alone (Palmer *et al.*, Br. Med. J., 286, 624-626, 1983). In the United States, the reports of undesirable events linked to the T and Td vaccines administered to individuals over 7 years old have recently been analyzed (Haber *et al.*, ICAAC, 1996). The Td vaccine exposed the individuals to local reaction, dyspnea, loss of consciousness or a convulsion more than the T vaccine did.

With regard to poliomyelitis, two vaccines were developed in the 1950s: the injectable vaccine (IPV), which is inactivated with formaldehyde, developed by Salk (Plotkin *et al.*, E.A. Vaccines, ed. Raven Press, 1994), and the oral vaccine (OPV), which is a live attenuated vaccine, developed by Sabin (Plotkin *et al.*, above). The IPV has enabled all countries to eliminate poliomyelitis, both wild-type and post-immunization poliomyelitis (Murdin *et al.*, Vaccine, 14, 735-746, 1996). The method of production and the composition of the IPV vary according to country. The three types of poliovirus are thus cultured on the VERO continuous cell line, and then purified and inactivated with formaldehyde. The respective immunizing activity thereof is assessed *in vitro* and *in vivo*. An ELISA measures the viral antigen content, expressed in international units: the WHO recommends, per dose of 0.5 ml, 40 IU, 8 IU and 32 IU (as determined by the Sigmoid method), respectively, for the D antigens of types 1, 2 and 3.

The IPV offers two pharmaceutical advantages: it is stable and does not require any specific logistic. It can sometimes be combined with other antigens.

Simultaneous immunization during infancy against poliomyelitis, tetanus and diphtheria has been common practice for some years throughout the world, indeed for decades in most developed countries. This practice has greatly contributed to considerably decreasing the number of cases and of deaths due to these three diseases. However, they have not disappeared. In developed countries, when they occur, the later in life they do occur, the more serious they are. Now, the immunity acquired by immunization attenuates with time. Since they do not benefit from a natural maintenance of this immunity, the teenagers and adults of these countries become vulnerable again. Maintaining the immunization coverage against each of

the three diseases throughout life is, from now on, an epidemiological obligation.

A need therefore exists for a novel vaccine against at least diphtheria, poliomyelitis and tetanus, which can be used in primary immunization and as an immunization booster, which would aim to confer protection against tetanus, poliomyelitis and diphtheria, and/or to prolong protection conferred initially during a primary immunization or a sensitization, and which would minimize, on this population, the undesirable effects induced by existing vaccines.

The injectable pediatric vaccines of the type TDPolio (T: tetanus toxoid; D: conventional dose of diphtheria toxoid; Polio: inactivated type 1, 2 and 3 polioviruses), for example the D.T.Polio® vaccine (PMsv S.A., France), cannot satisfy this need. Specifically, these types of vaccine are used mainly in pediatric primary immunization. They lack aluminum salts. They contain amounts of diphtheria toxoid which are too high, about 100 Lf/ml, for example, and they trigger undesirable reactions in adults (Björkholm *et al.*, Eur. J. Clin. Microb., 6, 637-640, 1987).

It is also not possible to envisage simply modifying an existing TDPolio vaccine in order to minimize the undesirable effects thereof on a population having undergone primary immunization. Specifically, a target of choice would, for example, be to reduce the amount of diphtheria toxoid therein. In this case, selecting the amounts of toxoid used conventionally in a Td vaccine (which is the only current reference), of the order of at least 4 Lf per ml, for example, would not be envisaged, but rather in adding more [sic], probably more than 20 Lf/ml, for example. In fact, in a conventional Td vaccine, the diphtheria toxoid is always adjuvanted with an aluminum salt, which reinforces the immunogenicity thereof. In a TDPolio vaccine, the fact of not having any adjuvant

implies reinforcing the toxoid dose with respect to that used in a Td vaccine.

For the same reasons, simply combining an existing vaccine of the injectable Td type, for example the vaccinol® or Td-Pur® vaccines (Chiron-Behring GmbH, Germany) or the Diftavax® vaccine (PMsv S.A., France), with a conventional vaccine against poliomyelitis (PMsv, France), could not produce a satisfactory solution either. Specifically, the choice of each constituent in such a vaccine, and also the dose thereof, are determinant in producing an optimal immune response and minimizing the undesirable effects.

Thus, for example, if novel antigens (Polio) are added to a Td. vaccine, the amount of aluminum salts is then likely to be insufficient to play an optimal adjuvant role. There would thus be a risk of the immunogenicity of such a vaccine being decreased. On the other hand, if the intention is to overcome this problem by increasing the amount of aluminum salts, there would be the risk, in parallel, of worsening the undesirable reactions linked to these salts (1.2 to 3 mg per ml in the Td vaccines; Gupta *et al.*, Vaccine, 13, 1263-1276, 1995).

Similarly, if novel antigens (Polio) are added to a Td vaccine, the relative load of each T or d antigen is also decreased. There would thus be a risk of the immunogenicity of such a vaccine also being decreased. On the other hand, if the intention is to overcome this problem by increasing the dose of each antigen, in particular that of the diphtheria toxoid, there would be a risk, in parallel, of worsening the undesirable reactions linked to this toxoid (Björkholm *et al.*, Eur. J. Clin. Microb., 6, 637-640, 1987).

The choice of each constituent of a vaccine of the type TdPolio, and also their dose, are therefore difficult to determine, and cannot be easily deduced from existing vaccines.

In addition, there is no immunization combination, against diphtheria, tetanus, poliomyelitis and whooping cough, or even also against hepatitis A and/or hepatitis B which is suitable for use as an immunization booster in adults or teenagers.

The present invention is thus aimed toward providing a vaccine having at least the basic Td Polio combination, which, while differing from the previous TDPolio, Td and Polio vaccines, has an immunogenicity comparable to these vaccines and, in addition, minimizes the undesirable effects thereof. The vaccine according to the invention has the advantage that it can be used in primary vaccination and in booster immunization.

Summary of the invention

To this end, the invention relates to a vaccine comprising:

- less than 1.2 mg per ml of aluminum salt, expressed with respect to the Al^{3+} atom,
- immunogenic antigens originating at least from the poliovirus, from *Corynebacterium diphtheriae* and from *Clostridium tetani*, and
- an amount of diphtheria toxoid used as an immunogenic antigen of *Corynebacterium diphtheriae* of between 4-16 Lf per ml.

The vaccine according to the present invention can be used in primary immunization and as a booster vaccine in a population which has undergone primary vaccination or is sensitized. The Applicant has demonstrated that the vaccine as defined above is particularly suitable for use as an immunization booster.

The Applicant has demonstrated, surprisingly, that the vaccine as defined above makes it possible to minimize the reactogenic and/or allergic effects induced by the constituent antigens.

According to another subject, the present invention relates to a method for immunizing against at

least the poliovirus, *Corynebacterium diphtheriae* and *Clostridium tetani*, comprising the administration of a vaccine as defined above.

Finally, the invention also relates to a pharmaceutical kit comprising at least 2 injectable doses of a vaccine according to the present invention.

Detailed description of the invention

In the context of the present invention, the expression "a population which has already undergone primary immunization or is already sensitized" is intended to mean adult, teenage or young individuals having already been immunized against at least the poliovirus, *Corynebacterium diphtheriae* and/or *Clostridium tetani*, or individuals having already been in contact with one at least of the poliovirus, *Corynebacterium diphtheriae* and *Clostridium tetani* microorganisms; the preferred population consisting of teenagers and adults, and more particularly elderly individuals.

Preferably, the origin of the *Corynebacterium diphtheriae* and *Clostridium tetani* antigens is the toxins thereof, which are detoxified with formaldehyde and then purified. The techniques for detoxifying and for purifying these toxins have been well known for decades and are incorporated into the description of the present invention by way of reference, such as those described by Leong *et al.*, Science, 220, 815-517 [sic], 1983; Ramon G., Ann. Inst. Pasteur, 38, 1-105, 1924; or Raynaud *et al.*, Ann. Inst. Pasteur, 96, 60-71, 1959; or by Bizzini *et al.*, Eur. J. Biochem., 17, 100-105, 1970, for example. The detoxified analogs which can be produced by genetic engineering are also included in the present invention.

Similarly, the poliovirus antigens can simply consist of one or more types of inactivated poliovirus (see hereinafter) and/or of purified immunogenic antigens of the poliovirus, such as those described by Delpeyroux *et al.*, 70, 1065-73, 1988; EP323861 (Pasteur

Institut); EP86707 (Pasteur Institut); or in EP65924 (Pasteur Institut), for example.

In order to obtain inactivated polioviruses, it is possible to culture them on VERO cell lines, purify them and then detoxify them with formaldehyde, for example. The techniques for culturing and for detoxifying the poliovirus have been well known for decades and are also incorporated into the description of the present invention by way of reference, such as those described in WO9800167 (Connaught); Dulbecco, Nature, 376, p.216, 1995; and Cohen, Acta Leiden, 56, 65-83, 1987, and by Salk, Dev. Biol. Stand., 47, 247-55, 1981, for example.

Preferably, inactivated type 1 (MAHONEY strain), 2 (MEF 1 strain) and 3 (Saukett strain) polioviruses are used.

The amount of diphtheria toxoid used per immunization dose as an immunogenic antigen of *Corynebacterium diphtheriae* should be between 4 and 16 Lf per ml, preferably 6-14 Lf, in particular about 10 Lf per ml, for example. This amount makes it possible to ensure optimal immunogenicity, while at the same time minimizing the undesirable effects, such as the reactogenic or allergic reactions to the antigens, for example.

Similarly, the amount of tetanus toxoid per immunization dose as immunogenic antigen of *Clostridium tetani* is between 6 and 30 Lf per ml, preferably 8-20 Lf, in particular about 10 Lf per dose, for example. This amount makes it possible to ensure optimal immunogenicity, while at the same time minimizing the undesirable effects.

In order to measure the amount of diphtheria or tetanus toxoid (Lf), the floculation assay described by Lyng (J. Biol. Stand., 15, 27-37, 1987; or Biologicals, 18, 11-17, 1990), or the WHO (Manual for the production and the control of vaccines, BLG/UNDP/77.1 and BLG/UNDP/77.2) is used, taking into account, however, that the purity of the toxoid used is related to

1500-1800 Lf per mg of nitrogen for diphtheria, or 1000-1300 Lf per mg of nitrogen for tetanus.

By way of indication, in most manufacturings, the purity of the purified diphtheria toxoid is generally about 1700-1800 Lf per mg of nitrogen (internal source). Similarly, for the tetanus toxoid, this purity is about 1200-1400 Lf (internal source).

With regard to the inactivated polioviruses used as immunogenic antigens, an immunization dose according to the invention can comprise 60-120 IU/ml of D antigen of the type 1 poliovirus, 8-30 IU/ml of D antigen of the type 2 poliovirus and/or 16-80 IU/ml of D antigen of the type 3 poliovirus, for example. The International Units are determined according to the Sigmoïd method.

In a particular embodiment of the present invention, the vaccine according to the invention can comprise, in addition to the basic TdPolio combination described above, 0.1 to 60 µg/ml of purified *B. pertussis* antigens, preferably *B. pertussis* toxoid (PT) and F-HA; 0.1 to 40 µg/ml of the hepatitis B HBs antigen; and/or 0.1 to 40 µg/ml of inactivated hepatitis A virus or an antigen thereof, for example.

The *Bordetella pertussis* toxin, including the detoxified analogs produced by genetic engineering, can be produced in different ways. For example, a *B. pertussis* strain can be cultured according to conventional methods (Sekura *et al.*, J. Biol. Chem., 258, 14647-14651, 1993), the toxoid can be isolated by adsorbing the culture medium onto an Affi-Gel Blue® (BioRad Lab, US) column and then eluting it with a solution rich in salts (for example, 0.75 M of magnesium chloride), and then, after having removed the salts, this eluent can then again be adsorbed onto a fetuin-Sepharose affinity column (composed of fetuin linked to cyanogen bromide) and then eluted with a 4M solution of a magnesium salt. The *B. pertussis* toxin can then be detoxified using glutaraldehyde according to a modified method from Munoz *et al.* (Infect. Immun.,

33, 820-826, 1981), as described in patent application PCT/EP97/05378 (PMsv). Many other methods are also available to those skilled in the art, such as those of Irons *et al.* (Biochem. Biophys. Acta. 580, 175-185, 1979), or those described in patents US 4705686 and EP 336736, and are incorporated into the description of the present invention by way of reference.

The F-HA can be purified from the culture supernatant essentially by the process described by Cowell *et al.* (Infect. And Immun., 41, 1, 313-320, 1983). Growth promoters, such as methylated beta-cyclodextrins, can be used to increase the F-HA yield in the supernatant. The culture supernatant is loaded onto a hydroxyapatite column. The F-HA is adsorbed onto the column, but not the PT. The column is very thoroughly washed with Triton X-100 in order to eliminate the endotoxin. The F-HA is then eluted using 0.5M NaCl in 0.1M sodium phosphate and, if necessary, passed over a fetuin-Sepharose column in order to eliminate the residual PT. A further purification can comprise being passed through a Sepharose CL-6B column. An alternative can comprise purifying the F-HA through the use of monoclonal antibodies directed against the antigen, in which the antibodies are attached to a CNBr-activated affinity column. The F-HA can also be purified using chromatography on perlite, as described in patent EP 336 736.

A method suitable for purifying the F-HA is also described in Example 3 of patent EP 0242 302. In the context of the present invention, the F-HA is prepared according to this method.

A suspension of the hepatitis B antigen can be produced according to the method described in patent EP 273811 (Pasteur Vaccins), in which antigenic hepatitis B surface particles are produced by expression using a culture of CHO cells transfected with a plasmid carrying the HBsAg gene so as to release the antigenic surface particles into the culture medium. Other techniques are well known to those

skilled in the art, and are incorporated into the description of the present invention by way of reference, such as those described in EP 864649, UE 56711 or IE 48665, for example.

Similarly, an inactivated hepatitis A virus can be produced according to the protocol of Flehmig *et al.* (Viral Hepatitis and Liver Disease, 87-90, 1988; J. Med. Virol., 22, 7-16, 1987). Other techniques are well known to those skilled in the art, and are incorporated into the description of the present invention by way of reference, such as those described by Wang *et al.*, Vaccine, 13, 835-40, 1995; Shevtsova *et al.*, Zh Mikrobiol Epidemiol Immunobiol., 2, 55-90, 1995; and Richtmann *et al.*, J. Med. Virol., 48, 147-50, 1996; or in EP 199480; IE 48399; or IE 50191, for example.

The vaccine according to the invention comprises one or more adjuvants chosen from adjuvants recognized as such, in particular all aluminum salts, such as aluminum phosphates and hydroxides; N-acetylmuramyl-L-alnanyl-D-isoglutamyl-L-alanine-2-[1,2-dipalmitoyl-sn-glycero-3-(hydroxyphosphoryloxy)] (see Sanchez-Pescador *et al.*, J. Immu., 141, 1720-1727, 1988); molecules derived from *Quillaja saponaria*, such as Stimulon® (Aquila, US); Iscoms® (CSL Ltd., US); all molecules based on cholesterol and analogs, such as DC Chol® (Targeted Genetics); the glycolipid Bay R1005® (Bayer, Germany); *Leishmania brasiliensis* antigens, such as LeIF (technical name), available from Corixa Corp. (US), and polymers of the polyphosphazene family, such as Adjumer (technical name), available from the "Virus Research Institute" (US), for example. The vaccine according to the present invention contains less than 1.2 mg/ml, preferably 0.70 mg/ml, of aluminum salt, expressed with respect to the Al^{3+} atom.

It may be noted that, with respect to conventional TDPolio vaccines, such as D.T. Polio® (PMsv, France), the present invention proposes, for the first time, the addition of an adjuvant, and in

particular an aluminum salt such as aluminum hydroxide for example.

Against all expectations, the vaccine according to the present invention can comprise an amount of aluminum salt which is lower than those encountered in all the conventional Td vaccines, such as Td-Pur® or Diftavax®, whereas it might have been considered necessary to increase the load thereof subsequent to the addition of the inactivated polioviruses. A vaccine according to the invention comprises less than 1.2 mg/ml of an aluminum salt, preferably less than 0.8 mg/ml. The amount of aluminum salt is always expressed with respect to the aluminum atom (Al^{3+}), which corresponds to the only method which can be used in the vaccines field. All the amounts of aluminum salts to which reference is made in the present application are therefore expressed in this way.

A vaccine according to the present invention can comprise other constituents, for instance preserving agents such as 2-phenoxyethanol and/or formaldehyde, etc., for example.

The various formulations selected can be one of those described above, for example, in particular those comprising other antigens originating from *Bordetella pertussis*, from hepatitis A or from hepatitis B.

The vaccine according to the invention can be in the form of an injectable suspension, slightly opalescent due to the presence of an insoluble aluminum salt. The immunization dose is preferably about 0.5 ml, contained in a prefilled glass syringe. The administration is carried out via the deep subcutaneous route or via the intramuscular route, preferably via the intramuscular route, for example in one of the deltoid muscles.

In a particular embodiment of the present use, several doses of vaccine are intended to be injected separately into the same individual in a period of time of between 10 days and 3 months, so as to promote an optimal immune reaction and so as to minimize the

reactogenic and/or allergic effects of these antigens. For a primary immunization, the vaccine according to the present invention is preferably administered in 3 doses, the first two doses being administered 1 to 2 months apart, the third dose being separated from the second injection by a period of 6 to 12 months. For a booster immunization which can be used on a population which has undergone primary immunization or is sensitized, the vaccine according to the present invention is administered in one dose, or 2 doses separated by at least 1 month.

The vaccine according to the invention can advantageously be used in a booster immunization in which the primary immunization has been carried out using an oral vaccine against poliomyelitis.

Preferably, a dose of 0.5 ml comprising 4 to 16 Lf/ml of diphtheria toxoid, preferably 10 Lf/ml; 6 to 30 Lf/ml of tetanus toxoid, preferably 20 Lf/ml and preferably 40 IU, 8 IU and 32 IU (Sigmoid method), respectively, of the D antigens of the polioviruses 1, 2 and 3 are used, both in primary immunization and in booster immunization. The amount of aluminum salt present is preferably 0.70 mg/ml, expressed with respect to the Al^{3+} atom.

The vaccine according to the invention makes it possible to decrease the undesirable effects induced by the existing TDPolio and Td vaccines when they are injected. It is thus possible to observe a decrease in local reactions such as numbness of the limbs, sweating, fever, pain associated with red blotches, nodules, indurations and/or ecchymoses, and a decrease in dyspnea, losses of consciousness or convulsions, for example. These reactions can be classified among allergic, or even reactogenic, reactions to the antigens and/or to certain other compounds of the vaccine, such as the aluminum salt. The Applicant has thus shown that the vaccine according to the present invention is particularly suitable for booster immunization, in particular in adult individuals.

The present invention is described in greater detail in the examples given hereinafter. The percentages are given by weight unless otherwise indicated. It goes without saying, however, that these examples are given by way of illustration of the subject of the invention, of which they in no way constitute a limitation.

Example 1 Immunogenicity of the TdPolio with various doses of diphtheria toxoid

Diphtheria toxoid (d) is prepared by culturing the IM 1514 N3S strain in an IMD medium for 15 to 18 h at 36°C, centrifuging the medium, clarifying it, concentrating it by ultrafiltration, detoxifying it at 37°C for 4 weeks in the presence of 6/1000 of formalin, and then purifying the toxin to reach a purity of about 1700 Lf per mg of nitrogen.

In parallel, the tetanus toxoid (T) is prepared by culturing the Harvard strain No. 49205 IM 1472C in a Massachusetts medium at 35°C for 4 days, adding NaCl and sodium citrate thereto, centrifuging the medium, concentrating it by ultrafiltration, detoxifying it for 2 weeks at 35°C in the presence of 5.5/1000 of formalin and 5 g/l of sodium bicarbonate, and then purifying the toxin to reach a purity of about 1200 Lf per mg of nitrogen.

Similarly, the inactivated type 1 (MAHONEY strain), 2 (MEF 1 strain) and 3 (Saukett strain) polioviruses are prepared according to the Salk method.

The 5 immunogenic antigens above are then mixed with aluminum hydroxide, 2-phenoxyethanol, formaldehyde, Hanks medium 199 without phenol red, and water. To do this, the aluminum gel is sterilized in the presence of water, the pH is adjusted between 5.6 and 6, the PDT (2 or 8 Lf/dose) and PTT (10 Lf/dose) and the Hanks medium 199 are added sequentially, the pH is adjusted to 6-6.9, the three types of poliovirus are added, and then the 2-phenoxyethanol and formaldehyde

are added and, optionally, the pH is adjusted between 6.8 and 7.

The final product of 0.5 ml will be used as TdPolio vaccine and contains a minimum of 2 IU of diphtheria toxoid, a minimum of 20 IU of purified tetanus toxoid, 40 IU, 8 IU and 32 IU of D antigen (values as measured by the Sigmoid method), respectively, for the inactivated type 1, 2 and 3 polioviruses, 0.35 mg of aluminum hydroxide, expressed with respect to the aluminum atom Al^{3+}, 2.5 µl of 2-phenoxyethanol and 12.5 µg of formaldehyde, the rest consisting of Hanks medium 199 without phenol red, and of water.

The main objective of a phase I study has been to validate the clinical and biological tolerance of the first administration of an adsorbed TdPolio vaccine. With this aim, 31 healthy volunteer adults were recruited. Three batches of the vaccine, of 0.5 ml each, were used. They differed only in the amount of purified diphtheria toxoid described above: <u>2 Lf, 5 Lf and 8 Lf per dose</u>. They were attributed sequentially in a proportion of 10 individuals per batch. Each individual was immunized with one dose from one batch, injected into a deltoid muscle.

No general reaction was reported in the immunized groups, with the batches at 2, 5 and 8 Lf. At least one local reaction was reported during the first week in 8 individuals of the group immunized with the batch at 2 Lf of PDT, in 6 individuals of the group immunized with the batch at 5 Lf of PDT and in 8 individuals of the group immunized with the batch at 8 Lf of PDT; it always involved pain associated with some red blotches, nodules, indurations and/or ecchymoses. All the reactions disappeared without treatment and did not alter the every-day life of the individuals. No reaction occurred beyond the first week. No serious undesirable event was declared by the investigators.

The secondary objective of this study was to evaluate the immunogenicity of the first three batches of the adsorbed vaccine. The results show that the immune response to the five antigens was excellent for the three batches. Despite high initial titers due to the young age of the individuals and to recent immunizations, a booster effect was obtained for each antigen.

Example 2 Immunogenicity in young adults

The immunogenicity and the innocuity of a TdPolio vaccine was determined during a clinical trial in 508 young adults.

For each individual, a dose of 0.5 ml of a Td (Diftavax®) or TdPolio vaccine was injected into the left deltoid muscle, and a dose of 0.5 ml of the vaccine against poliomyelitis (VPI®, PMsv) or a placebo was injected into the right deltoid muscle. A dose of adsorbed Td vaccine contains tetanus toxoid (activity ≥ 20 IU), diphtheria toxoid (activity ≥ 2 IU) and aluminum hydroxide (≤ 1.25 mg). The IPV vaccine contains the D antigens of the type 1 (40 IU), 2 (8 IU) and 3 (32 IU) polioviruses. The placebo has the same composition as the IPV with the only difference being that it does not contain any D antigens. The TdPolio vaccine has the same composition as that described in example 1 (5 Lf of PDT per dose).

The anti-diphtheria, tetanus and poliomyelitis antibodies were determined from the sera of the individuals, using the ELISA assay.

The results show that, before immunization, virtually all the individuals were seropositive with regard to the PDT (99.2% have a titer ≥ 0.01 IU/ml: possible protection; and 92.6% have a titer ≥ 0.1 IU/ml: assured protection). One month after immunization with the TdPolio, 99.6% of the individuals had an antibody titer ≥ 0.1 IU/ml and 82.4% had a titer ≥ 1 IU/ml (long term protection). In total, 17.4% of the individuals showed seroconversion.

Similarly, the majority of the individuals were seropositive with regard to the tetanus toxoid (99.6% having a titer ≥ 0.01 IU/ml: possible protection; and 98.4% having a titer ≥ 0.1 IU/ml: assured protection). One month after immunization with the TdPolio, 100% of the individuals have an antibody titer ≥ 0.1 IU/ml, and 99.2% have a titer ≥ 1 IU/ml (long term protection). In total, 25.2% of the individuals showed seroconversion.

With regard to protection against poliomyelitis, the majority of the individuals were seropositive with regard to the three types of poliovirus. In total, 99.2% of the individuals had an antibody titer ≥ 5 for type 1, 100% for type 2 and 97.6% for type 3. One month after immunization with the TdPolio, 100% of the individuals have a titer ≥ 5 for all the poliovirus types, with a minimum titer of 120 for type 1, 160 for type 2 and 80 for type 3. In total, 63.1% of the individuals already having a high antibody titer show seroconversion.

When the results obtained with the TdPolio and those obtained when combining the Td and VPI® reference vaccines are compared, an equivalent antibody response is obtained.

Example 3 Innocuity and tolerability in young adults

The innocuity and tolerability of a TdPolio vaccine were determined in 1742 young adults. Each one of these individuals received a dose of 0.5 ml of the vaccine shown in example 1 (PDT: 5 Lf per dose), injected into the left deltoid muscle. The effects were evaluated 15 minutes after immunization. The local, regional and systemic effects were evaluated during the month following immunization.

The common immediate effect is the appearance of a red blotch at the point of injection (0.34% of the individuals) and pain (0.11%).

The common local effects during the months following immunization are as follows for 66% of the

individuals: pain (64.41%), red blotch at the point of injection (9.13%) and subcutaneous nodules (3.33%). These undesirable effects appear on the first 3 days following immunization and last 2 to 3 days. 0.86% of the individuals also reported events of edema, of inflammation, of migraine, of numbness of the arm, of involuntary muscle contractions and paraesthesia.

The common systemic effects during the month following immunization are as follows for 18% of the individuals: headaches (10.5%), nausea or vomiting (2.75%) and malaise (2.41%). No event of generalized urticaria or itching was reported. These events appear in the first 3 days following immunization and last 2 to 3 days. Only 0.23% of the individuals have a temperature which exceeds ≥ [sic] 40°C during the first 3 days following immunization.

Example 4 Innocuity and tolerability of the TdPolio *versus* Td + Polio in young individuals

The TdPolio vaccine described in example 2 shows excellent tolerability during the trial on 508 young adults (example 2). The results are, moreover, comparable to those obtained during the trial described in example 3.

In subtracting the percentage of individuals having had at least one local or regional sign at the site of injection of the placebo (14.8%) from those observed at the site of injection of the Polio vaccine (36.5%), and then adding this percentage to that observed at the site of injection of the Td vaccine (66.7%), a difference of approximately 8% in the appearance of undesirable events (88.4% vs 80.5%) is observed. The TdPolio vaccine is therefore tolerated better than the simultaneous administration of the existing vaccines.

Example 5 Immunogenicity in individuals over 40 years old

The immunogenicity and innocuity of a TdPolio vaccine were determined during a clinical trial on 113 individuals over 40 years old (40 to 78 years old). All the individuals had received primary immunization (3 doses in one year) against diphtheria, tetanus and poliomyelitis, the last immunization dating back 32 years (minimum 15 years), 28 years (minimum 10 years) and 28 years (minimum 10 years), respectively.

The individuals received an injection of a dose of 0.5 ml of the TdPolio described in example 1 (PDT 5 Lf per dose) in the right deltoid muscle, and 28 days later, a second injection in the left muscle. The anti-diphtheria, tetanus and poliomyelitis antibodies were evaluated from the sera of the individuals, using the ELISA assay.

The results show that only 50% of the individuals were initially protected against tetanus, thus demonstrating the decline in the immunity. 83% of the individuals were initially seropositive against the type 1, 2 and 3 polioviruses. On day 28 following the first immunization, the proportion of individuals protected against diphtheria, tetanus and the type 1, 2 and 3 polioviruses had risen to 80.5%, 97.3% and 100%, respectively. On day 56 following the administration of the second dose, the proportion of individuals protected against diphtheria, tetanus and the type 1, 2 and 3 polioviruses had risen to 93.7%, 100% and 100%, respectively.

<u>Example 6</u> Innocuity and tolerability of the TdPolio *versus* Td + Polio in individuals over 40 years old

The innocuity and tolerability of the TdPolio vaccine described in example 5 were evaluated. The results show a tolerability profile similar to that obtained with the Td + Polio combination. <u>However, the</u>

TdPolio vaccine induces fewer serious undesirable events (less than 1.8 per 1000 injections).

Example 7 d.T.Polio.PT.F-HA booster vaccine for adults

An immunization suspension of 0.5 ml is prepared, with or without preserving agent, consisting of 5 Lf of diphtheria toxoid (having a purity of 1700 Lf per mg of nitrogen), 5 Lf of tetanus toxoid (having a purity of 1200 Lf per mg of nitrogen), 40 IU, 8 IU and 32 IU of D antigen (values as determined by the Sigmoid method) respectively, for the inactivated type 1, 2 and 3 polioviruses, 6 µg/ml of purified *Bordetella pertussis* toxoid (PT) and 6 µg/ml of *Bordetella pertussis* F-HA, and 0.35 mg of aluminum hydroxide, the rest consisting of Hanks medium 199 without phenol red, and of water.

2.5 µl of 2-phenoxyethanol and 12.5 µg of formaldehyde can also be added as a preserving agent.

The diphtheria and tetanus toxoids, and also the inactivated polioviruses, were prepared as described in example 1.

The *B. pertussis* toxoid is prepared according to the method of Sekura *et al.* (J. Biol. Chem., 258, 14647-14651, 1993), detoxified according to a modified protocol from Munoz *et al.* (Infect. Immun., 33, 820-826, 1981), and then preadsorbed on an aluminum gel. The *B. pertussis* F-HA is prepared using the method described in example 3 of EP 0242 302, and then preadsorbed on an aluminum salt.

The immunogenic antigens above are then mixed with aluminum hydroxide and water, and where appropriate, with the preserving agents. To do this, the aluminum gel is sterilized in the presence of water, the pH is adjusted between 5.6 and 6, the PDT, the PTT and the *B. pertussis* PT and F-HA are added sequentially, the pH is adjusted to 6.8-7, the Hanks medium 199 and the three types of poliovirus are added,

and then, where appropriate, the 2-phenoxyethanol and the formaldehyde are added.

<u>Example 8</u> d.T.Polio.PT.F-HA.HBs booster vaccine for adults

An immunization suspension of 0.5 ml is prepared, without preserving agent, consisting of 5 Lf of diphtheria toxoid (having a purity of 1700 Lf per mg of nitrogen), 5 Lf of tetanus toxoid (having a purity of 1200 Lf per mg of nitrogen), 40 IU, 8 IU and 32 IU of D antigen, respectively, for the inactivated type 1, 2 and 3 polioviruses, 6 μg/ml of purified *Bordetella pertussis* toxoid (PT), 6 μg/ml of *B. pertussis* F-HA, 5 μg/ml of hepatitis B HBs antigen and 0.35 mg of aluminum hydroxide, the rest consisting of Hanks medium 199 without phenol red, and of water.

The diphtheria and tetanus toxoids, and also the inactivated polioviruses, were prepared as described in example 1. The *B. pertussis* PT and F-HA were prepared as described in example 7.

The HBs antigen was prepared according to the method described in patent EP 273 811 (Pasteur Vaccins). It is stabilized by preadsorption on an aluminum gel.

The immunogenic antigens above are then mixed with aluminum hydroxide and water. To do this, the aluminum gel is sterilized in the presence of water, the pH is adjusted between 5.6 and 6, the PDT, the PTT and the *B. pertussis* PT and F-HA are added sequentially, the pH is adjusted to 6.8-7, the Hanks medium 199 and the three types of poliovirus are added, the pH is adjusted to 6.8 if necessary and the HBs is added.

<u>Example 9</u> d.T.Polio.PT.F-HA.HBs.HA booster vaccine for adults

An immunization suspension of 0.5 ml is prepared, without preserving agent, consisting of 5 Lf of diphtheria toxoid (having a purity of 1700 Lf per mg of nitrogen), 5 Lf of tetanus toxoid (having a purity of 1200 Lf per mg of nitrogen), 40 IU, 8 IU and 32 IU of D antigen, respectively, for the inactivated type 1, 2 and 3 polioviruses, 6 μg/ml of purified *Bordetella pertussis* toxoid (PT), 6 μg/ml of *B. pertussis* F-HA, 5 μg/ml of hepatitis B HBs antigen, 5 μg/ml of inactivated hepatitis A virus and 0.35 mg of aluminum hydroxide, the rest consisting of Hanks medium 199 without phenol red, and of water.

The diphtheria and tetanus toxoids, and also the inactive polioviruses, were prepared as described in example 1. The *B. pertussis* PT and F-HA were prepared as described in example 7. The HBs antigen was prepared as described in example 8.

The inactivated hepatitis A virus is prepared according to the method of Flehmig *et al.* (above).

The immunogenic antigens above are then mixed with aluminum hydroxide, the preserving agents and water. To do this, the aluminum gel is sterilized in the presence of water, the pH is adjusted between 5.6 and 6, the PDT, the PTT and the *B. pertussis* PT and F-HA are added sequentially, the pH is adjusted to 6.8-7, the Hanks medium 199 and the three types of poliovirus are added, the pH is adjusted to 6.8 if necessary, and then the HBs and the inactivated hepatitis A virus are added sequentially.